

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460



OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

MEMORANDUM

Date: August 8, 2012

SUBJECT: Naphthalene, Immunotoxicity study in Mice

PC Code: 055801

Decision No.: 374031

Petition No.: N/A

Risk Assessment Type: N/A

TXR No.: 0056395

MRID No.: 48792701

DP Barcode: 401779

Registration No.: N/A

Regulatory Action: N/A

Submission No.: N/A

CAS No.: 91-20-3

40 CFR: N/A

FROM: Yung G. Yang, Ph.D.
Risk Assessment Branch VI
Health Effects Division (7509 P)

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THROUGH: Felecia Fort, Chief
Risk Assessment Branch VI
Health Effects Division (7509 P)

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TO: Molly Clayton
RMIB 3
Pesticide Re-Evaluation Division (7508P)
And
Michael Metzger, Chief
Risk Assessment Branch V
Health Effects Division (7509P)

I. CONCLUSIONS

The immunotoxicity study in mice for Naphthalene (MRID 48792701) has been reviewed. It is classified as acceptable/guideline and satisfies guideline requirements for an immunotoxicity study (OPPTS 870.7800).

II. BACKGROUND and ACTION REQUESTED

An immunotoxicity study on Naphthalene (MRID 48792701) has been submitted. RAB VI was asked to review and prepare a DER for this study.

III. RESULTS AND DISCUSSION

The immunotoxicity study in mice for Naphthalene (MRID 48792701) has been reviewed. The DER is attached and an executive summary is as follows:

EXECUTIVE SUMMARY: In an immunotoxicity study (MRID 48792701), Naphthalene (99.28 % a.i., batch number 110541) was administered to female CD-1 mice (10/dose) by oral (gavage) at dose levels of 0 (control group), 25, 100 or 350 mg/kg/day for 4 weeks. On Day 25 (4 days prior to necropsy), all animals in all groups received a single intravenous dose of sheep red blood cells (4×10^8 cells/animal; dose volume 0.2 mL/animal) into the tail vein. The positive control group consisting of 8 female mice was administered cyclophosphamide 20 mg/kg by oral gavage on Days 22-26.

Parameters such as, clinical condition, body weight, food and water consumption, organ weights, and immunotoxicity related to the treatment were monitored. At necropsy on Day 29, the thymus and spleen from animals were removed and weighed. T-lymphocyte dependent antibody response (TDAR) to sheep red blood cells (SRBC) was measured by using a modified Jerne Plaque Forming Cell (PFC) assay.

Animals at 350 mg/kg/day had fast respiration, piloerection, lacrimation, under activity and partial closure of the eyelids; mainly during the first week and intermittently during the remainder of the treatment period. One female in this high dose group died on Day 14 and was considered treatment related. Body weight loss occurred between Days 1 and 4 in animals receiving 100 or 350 mg/kg/day, but later gained weight similar to or higher than control group. This resulted in reduction of weight gain ($\downarrow 65\%$) over the 4 week treatment period for females receiving 350 mg/kg/day when compared to the control. There was no effect of treatment on food intake. Water consumption was consistently higher than control in the high dose group. There were lower spleen and thymus weights in high dose group when compare to the vehicle control group.

The systemic toxicity NOAEL is 100 mg/kg bw/day. The systemic toxicity LOAEL is 350 mg/kg bw/day based on reduced body weight, spleen and thymus weights.

There were no statistically significant treatment-related effects on the anti-SRBC PFC response (PFC/ 10^6 cells and PFC/spleen) in all treated groups when compare with the control. There was a significant decrease in cells/spleen ($p=0.01$) in high dose group, this correlated with a decrease in spleen weights. A high inter-individual variability was noted in all the treatment groups as well as in the control group. Evaluation of individual animal data of this study did not show any trend or distribution that would demonstrate significant suppression of anti-SRBC PFC response. Animals in positive control group, showed a statistically significant ($p<0.001$) decrease of the anti-SRBC PFC response to a

challenge with SRBCs. This confirmed the ability of the test system to detect immunosuppressive effects and confirmed the validity of the study design.

The Natural Killer (NK) cells activity was not evaluated in this study. Decreased spleen and thymus weights were observed at 350 mg/kg/day group. However, the decrease may be due to secondary effect of general toxicity at the high dose. The toxicology database for Naphthalene does not reveal any evidence of treatment related effects on the immune system. The overall weight of evidence suggests that the chemical does not directly target the immune system. Under HED guidance a NK cell activity assay is not required at this time.

The immunotoxicity NOAEL is 350 mg/kg/day (the highest dose tested). The immunotoxicity LOAEL is not established.

This immunotoxicity study is classified **acceptable/guideline**; and satisfies the guideline requirement for an immunotoxicity study (OPPTS 870.7800) in the mouse.

EPA Primary Reviewer: Khin Swe Oo, MD, DABT.**Signature:** **Toxicology and Epidemiology Branch, Health Effects Division (7509P)****Date:** 8/8/2012**EPA Secondary Reviewer:** Yung G. Yang, Ph.D.**Signature:** **Risk Assessment Branch 6, Health Effects Division (7509P)****Date:** 8/8/2012

Template version 09/11

TXR #: 0056395**DATA EVALUATION RECORD****STUDY TYPE:** 4 Week Oral (gavage) Immunotoxicity Study - Mouse
OPPTS 870.7800**PC CODE:** 055801**DP BARCODE:** D401779**TEST MATERIAL (PURITY):** Naphthalene (99.28% w/w a.i.)**SYNONYMS:** LX 1298-01; Camphor Tar; Moth balls.**CITATION:** Chambers P., (2012). Naphthalene: 4 Week Oral Gavage Immunotoxicity Study in the Female CD 1 Mouse. Huntingdon Life Sciences Ltd., Wolley Rd. Alconbury, Huntingdon, Cambridgeshire, PE28 4HS, England. Project MCW0027, April 4, 2012. MRID 48792701. Unpublished.**SPONSOR:** RECOCHEM, INC., 850 Montee de Liesse Road, Montreal, Quebec, H4T 1P4, Canada.**EXECUTIVE SUMMARY:** In an immunotoxicity study (MRID 48792701), Naphthalene (99.28 % a.i., batch number 110541) was administered to female CD-1 mice (10/dose) by oral (gavage) at dose levels of 0 (control group), 25, 100 or 350 mg/kg/day for 4 weeks. On Day 25 (4 days prior to necropsy), all animals in all groups received a single intravenous dose of sheep red blood cells (4×10^8 cells/animal; dose volume 0.2 mL/animal) into the tail vein. The positive control group consisting of 8 female mice was administered cyclophosphamide 20 mg/kg by oral gavage on Days 22-26.

Parameters such as, clinical condition, body weight, food and water consumption, organ weights, and immunotoxicity related to the treatment were monitored. At necropsy on Day 29, the thymus and spleen from animals were removed and weighed. T-lymphocyte dependent antibody response (TDAR) to sheep red blood cells (SRBC) was measured by using a modified Jerne Plaque Forming Cell (PFC) assay.

Animals at 350 mg/kg/day had fast respiration, piloerection, lacrimation, under activity and partial closure of the eyelids; mainly during the first week and intermittently during the remainder of the treatment period. One female in this high dose group died on Day 14 and was considered treatment related. Body weight loss occurred between Days 1 and 4 in animals receiving 100 or 350 mg/kg/day, but later gained weight similar to or higher than control group. This resulted in reduction of weight gain ($\downarrow 65\%$) over the 4 week treatment period for females receiving 350 mg/kg/day when compared to the control. There was no effect of treatment on

food intake. Water consumption was consistently higher than control in the high dose group. There were lower spleen and thymus weights in high dose group when compare to the vehicle control group.

The systemic toxicity NOAEL is 100 mg/kg bw/day. The systemic toxicity LOAEL is 350 mg/kg bw/day based on reduced body weight, spleen and thymus weights.

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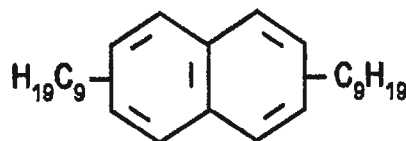
The immunotoxicity NOAEL is 350 mg/kg/day (the highest dose tested). The immunotoxicity LOAEL is not established.

This immunotoxicity study is classified **acceptable/guideline**; and satisfies the guideline requirement for an immunotoxicity study (OPPTS 870.7800) in the mouse.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS**A. MATERIALS:**

1. **Test material:** Naphthalene
Description: White crystalline powder
Lot/Batch #: 110541
Purity: 99.28% a.i.
Compound Stability: Stable at ambient temperature for 1 day and when refrigerated (2-8°C) for 15 days
CAS # of TGA: 91-20-3
Structure:



2. **Vehicle and/or positive control:** Corn oil was used for Groups 1-4 (vehicle control and Naphthalene treated animals). Cyclophosphamide (batch no. A 0277203 from Acros) was prepared using purified water.
3. **Test animals:**
- | | |
|---|---|
| Species: | Female Mice |
| Strain: | CrI:CD1 (ICR) |
| Age/weight when treatment started: | Approximately 43-50 days / 22.7 to 29.9 g. |
| Source: | Charles River (UK) Ltd. |
| Housing: | Animals were housed in pairs in cages made with polycarbonate and stainless steel mesh lid. Wood based material was used as bedding. |
| Diet: | Rat and Mouse No. 1 Maintenance Diet, <i>ad libitum</i> |
| Water: | Tap-water. <i>ad libitum</i> |
| Environmental conditions: | Temperature: 19-23°C
Humidity: 40-70%
Air changes: Own supply of filtered fresh air at +ve pressure, not recirculated.
Photoperiod: 12 hrs dark/12 hrs light |
| Acclimation period: | 3 weeks |

B. STUDY DESIGN:

1. **In life dates** – Study Start: April 21, 2011. End: April 4, 2012.
2. **Animal assignment:** Animals were assigned by using the sequence of cages in the battery, one animal at a time was placed in each cage and the procedure repeated until each cage held the appropriate number of animals. To facilitate the immunotoxicology investigations, the study was divided into two cohorts (cohort 1 comprised the first 6 females in Groups 1-4; cohort 2 comprised the remaining 4 females in Groups 1-4 and all Group 5/positive control females).

Table 1. Study design			
Group No.	Dose (mg/kg/day)	Volume dose (mL/kg)	No. of mice
1. Carrier Control	0	5	10
2. Naphthalene	25	5	10
3. Naphthalene	100	5	10
4. Naphthalene	350	5	10
5. Positive control	Cyclophosphamide 20mg/kg/day	10	8

Data obtained from page 15 in the study report.

* Positive control animals were administered 20 mg/kg cyclophosphamide by oral gavage on Days 22-26.

3. **Species and Gender Selection:** The Crl: CD1 (ICR) strain female mice were used because of the historical and positive and negative control data available in this laboratory.
4. **Dose selection:** The report stated that the dose selection was based on findings from 7-Day preliminary study in the CD-1 mouse using dose levels of 0, 150, 200, 350, and 500 mg/kg/day (Huntingdon Life Science Study no. MCW0028). The high dose caused weight loss and deteriorating clinical condition leading to the death of all animals in that group over the 7 day treatment period. Treatment with 350 mg/kg/day was tolerated over 7 days and was considered to be the Maximum Tolerated Dose level for naphthalene in CD-1 mice. The lower dose levels 25 and 100 mg/kg/day were selected as appropriate intervals to investigate any dose response.
5. **Diet preparation and analysis:** The formulations were prepared freshly each week by mixing the appropriate amount of the test substance, Naphthalene with corn oil and stored in a refrigerator (2-8°C).

The mean concentrations of Naphthalene in test formulations were within +10% to -15% of nominal concentrations. Individual results were within 2% of the mean value, confirming the precision of analysis. For homogeneity, the mean analyzed concentration for the samples remained within 4% of the initial time zero value and the coefficient of variation was less than 1%.

6. **Statistics:** Following statistical tests were used for body weight, organ weight, food and water consumption and PFC assay data. A parametric monotonic trend test, William's test was used for comparison of the means. If Bartlett's test for variance homogeneity was not significant at the 1% level, a parametric analysis, the F1 approximate test was applied. If the F1 approximate test was significant, Dunnett's test was performed. A non-parametric analysis, the H1 approximate test was performed if Bartlett's test was significant at the 1% level. Shirley's test was applied if the H1 approximate test for monotonicity of dose-response was not significant. Fisher's Exact tests (Fisher 1973) was used for cholinesterase data. Treatment groups were compared using pairwise comparisons of each dose group against the control group. One-way ANOVA was used for PFC data. Treated groups were

compared to control group using William's test (Williams 1971, 1972). For organ weight data, analysis of covariance was performed using terminal bodyweight as covariate.

C. METHODS:

1. **Observations:** Animals were visually inspected for general appearance twice daily during the exposure period.
2. **Body weight:** Animals were weighed twice during the acclimation period, on Day 1, and twice weekly during treatment, and the terminal body weight on the day of necropsy.
3. **Food consumption and compound intake:**
Food consumption values were recorded for the week before treatment started and each week throughout the treatment period.
4. **Water consumption:** For the week before treatment started and each week throughout the treatment, water consumption was recorded by weight (over a 3 day period on each occasion).
5. **Sacrifice and pathology:** On Day 29, animals were killed by carbon dioxide asphyxiation followed by subsequent exsanguinations. All animals were subject to a detailed necropsy, including external features, orifices, brain, pituitary gland, cranial nerves, neck, thorax, and abdominal viscera.
6. **Organ Weights:** Spleen and thymus weights were recorded at necropsy.
7. **Anti-SRBC plaque-forming-cell assay (PFC assay):** On Day 25 (4 days prior to necropsy), all animals in all groups received a single intravenous dose 0.2 mL by bolus injection of sheep red blood cells (2×10^9 cells/mL) into the tail vein. On Day 29, animals were sacrificed, spleens were harvested and put in the containers of Hank's Balanced Salt Solution and held on ice until processed for analysis. Splenocyte suspensions were prepared by mechanical dissociation and then used for the Plaque Forming cell (PFC) assay by using a modified hemolytic plaque assay of Jerne (Jerne NK et al, 1963, Holsapple, MP. 1995) which indicates the number of antibody-dependent lytic plaques.
8. **Natural Killer (NK) Cells Activity Assay:** Assay was not performed.

II. RESULTS:

A. Clinical Observations:

1. **Clinical signs of toxicity:** Animals at 350 mg/kg/day had fast respiration, piloerection, lacrimation, under activity and partial closure of the eyelids; mainly during the first week and intermittently during the remainder of the treatment period.
2. **Mortality:** One female in this high dose group died on Day 14 after having above

symptoms and signs and was considered treatment related.

- B. Body weight and weight gain:** Body weight loss occurred between Days 1 and 4 in animals receiving 100 or 350 mg/kg/day, but later gained weight similar to or higher than control group. This resulted in reduction of weight gain (↓65%) over the 4 week treatment period for females receiving 350 mg/kg/day compared to the control (Table 2).

Table 2. Mean Body Weight and Body Weight Gain (g)± SD				
	Dose Group (mg/kg/day)			
	0	25	100	350
Day 1	25.8±2.05	27.2±1.29	25.4±1.63	26.6±1.8
Day 8	25.4±1.96	27.6±1.62	25.0±1.18	25.6±1.88
Day 15	25.5±1.88	27.8±1.65	25.1±1.33	25.9±2.1
Day 29	28.0±2.1	30.0±1.76	27.3±1.83	27.5±2.07
Mean Body Weight Gain (g) ± SD				
Days 1-29	2.2±1.55	2.8±1.3	1.9±1.37	0.8*±1.17

n = 10 animals per dose group (high dose group had 9 only since one animal died on Day 14).

Information was obtained from pages 39 and 40 of the study report.

* < 0.05

C. FOOD CONSUMPTION AND COMPOUND INTAKE:

- 1. Food consumption:** There was no effect of treatment on food consumption
- 2. Compound consumption:** See Table 1.

- D. WATER CONSUMPTION:** Water consumption was consistently higher than control in the high dose group.

- E. Sacrifice and Pathology:** No treatment related gross pathology was observed.

- 1. Organ weight:** There were lower adjusted and body weight relative spleen and thymus weights in high dose group when compare to the vehicle control group (Table 3).

Table 3. Mean terminal body weight and thymus and spleen weights^a (g) ±SD					
Dose Group (mg/kg/day)	Terminal body weight (g)	Spleen		Thymus	
		Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Vehicle Control	27.6±1.9	0.154±0.023	0.556±0.1	0.0621±0.013	0.224±0.04
25	30.1*±1.7	0.142±0.04	0.467±0.1	0.0612±0.02	0.204±0.07
100	27.4±1.8	0.151±0.031	0.553±0.11	0.0603±0.01	0.221±0.04
350	27.6±2.0	0.107±0.018	0.386*±0.06	0.0462±0.011	0.168*±0.04

n = 10 animals per dose group (high dose group had 9 only since one animal died on Day 14).

^a Information was obtained from pages 43 - 44 of the study report

- 2. Histopathology – not performed.**

F. IMMUNOTOXICITY TESTS:

1. **Anti-SRBC PFC assay:** There were no statistically significant treatment-related effects on the anti-SRBC antibody response (PFC/ 10^6 viable cells or PFC/spleen) in all treated groups, when compare with the vehicle control group. There was a significant decrease in cells/spleen ($p=0.01$) at 350 mg/kg/day which correlated with a decrease in spleen weight (Table 4). There was a high inter-individual variability noted in all the treatment groups as well as in the control group. Evaluation of individual animal data of this study did not show any trend or distribution that would demonstrate significant suppression of anti-SRBC PFC response (Figures 1 and 2). The positive control group (cyclophosphamide 20 mg/kg/day for 5 days) resulted in a statistically significance decrease of anti-SRBC PFC responses in compared to the vehicle control group.

Table 4. Plaque Forming Cell Assay mean data			
Dose Group (mg/kg/day)	Cells/spleen ($\times 10^7$)	PFC/ 10^6 Spleen Cells	PFC/Spleen
Vehicle Control (0)	7.68 \pm 1.87	1046.3 \pm 528.2	76351 \pm 34097
25	6.99 \pm 2.54	961.5 \pm 380.7	74768 \pm 59410
100	7.69 \pm 3.02	1295.5 \pm 569.6	108701 \pm 77396
350	4.77* \pm 1.26	937.2 \pm 474.7	46929 \pm 29930
Positive Control ^b	4.62** \pm 1.81	117.5*** \pm 78.5	6030*** \pm 5308

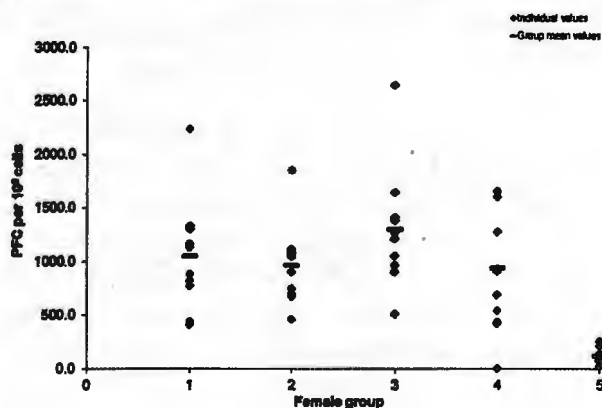
n = 10 animals per dose group; 9 animals in high dose group since one died on Day 14.

Information was obtained from pages 131 of the study report

^b=Cyclophosphamide (20 mg/kg/day) administered by oral gavage on Days 22-26.

Statistically significantly different from Group 1: * $p < 0.05$; *** $p < 0.001$

Figure 1: Plaque forming cell assay (PFC/ 10^6 viable spleen cells)



Key:

Group 1 = vehicle control group

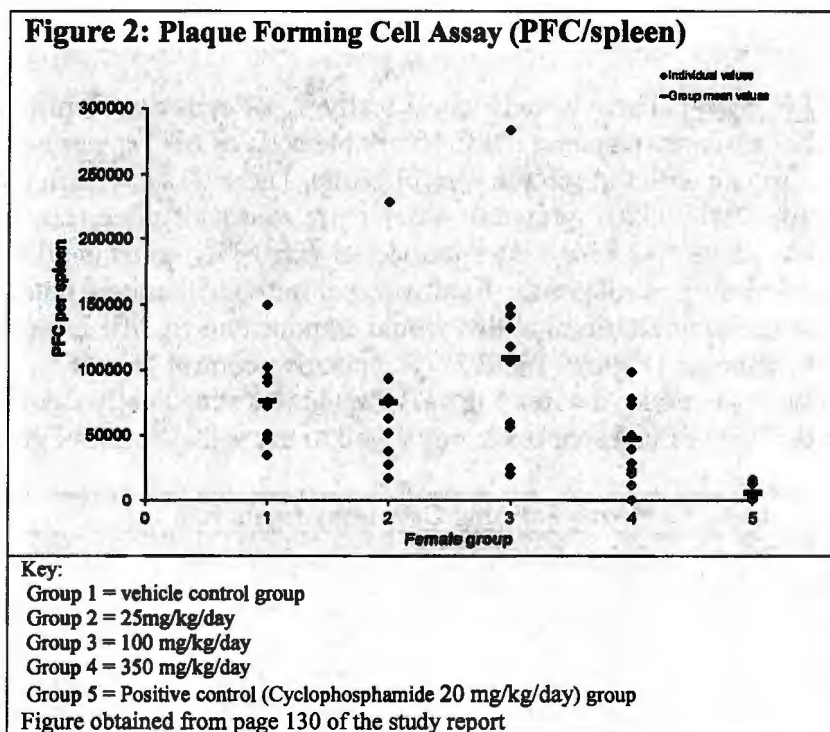
Group 2 = 25mg/kg/day

Group 3 = 100 mg/kg/day

Group 4 = 350 mg/kg/day

Group 5 = Positive control (Cyclophosphamide 20 mg/kg/day) group

Figure obtained from page 129 of the study report



2. Natural Killer (NK) Cell Activity Assay: Assay was not performed.

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS: It was concluded that administration of Naphthalene to female CD-1 mice for four weeks up to 350 mg/kg/day did not caused the suppression of the immune function.

B. REVIEWER COMMENTS: In an immunotoxicity study (MRID 48792701), Naphthalene (99.28 % a.i., batch number 110541) was administered to Female CD 1 mice (10/dose) by oral (gavage) at dose levels of 0 (control group), 25, 100 or 350 mg/kg/day for 4 weeks. On Day 25 (4 days prior to necropsy), all animals in all groups received a single intravenous dose of sheep red blood cells (4×10^8 cells/animal; dose volume 0.2 mL/animal) into the tail vein. The positive control group consisting of 8 female mice was administered cyclophosphamide 20 mg/kg by oral gavage on Days 22-26.

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the remainder of the treatment period. One female in this high dose group died on Day 14 and considered treatment related. Body weight loss occurred between Days 1 and 4 in animals receiving 100 or 350 mg/kg/day, but later gained weight similar to or higher than control group. This resulted in reduction of weight gain ($\downarrow 65\%$) over the 4 week treatment period for females receiving 350 mg/kg/day compared to the control. There was no effect of treatment on food intake. Water consumption was consistently higher than control in the high dose group. There were lower spleen and thymus weights in high dose group when compare to the vehicle control group.

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The immunotoxicity NOAEL is 350 mg/kg/day (highest dose tested). The immunotoxicity LOAEL is not established.

C. STUDY DEFICIENCIES: There were no major study deficiencies. The following minor deficiencies we noted, but would not change the conclusions of the study.

- The body weights, body weight gains, spleen and thymus weights of the positive control group (cyclophosphamide) were not reported.